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Cellular mechanisms of human muscle fatigue and disease-related loss of muscle function

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Cellular mechanisms of human muscle fatigue and disease-related loss of muscle function

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Abstract

A loss of skeletal muscle function and mass is a hallmark of several diseases resulting in a dismal life quality and poor prognosis. Impaired intracellular Ca^{2+}-handling has been proposed as an underlying cause of these manifestations. This thesis investigated the cellular mechanisms of human muscle fatigue and disease-related loss of muscle function and mass. Special focus was put on the contribution of altered intracellular Ca^{2+}-handling to these processes. The thesis makes three main contributions by: (*i*) providing mechanistic insight into the underlying causes of fatigue in human muscle fibres, (*ii*) elucidating the direct effects of vitamin D in human muscle, and (*iii*) implicating sphingomyelinase (SMase) activation in disease-related loss of muscle function and mass.

Study I examined single intact muscle fibres and differentiated muscle stem cells (myotubes) as experimental models of human skeletal muscle. Results revealed that single intact fibres could be manually dissected, enabling studies of Ca^{2+}-regulated processes and force generation. Major differences in intracellular Ca^{2+}-handling between myotubes and muscle fibres were discerned. In **study II**, the effects of vitamin D in human skeletal muscle were investigated. Data demonstrated a modulatory role of vitamin D on muscle stem cells, but did not support presence of the vitamin D receptor in muscle fibres. In **study III**, the cellular mechanisms of fatigue were examined in single intact fibres. Fatigue-induced force loss was caused by a reduction in sarcoplasmic reticulum (SR) Ca^{2+} release, while a decrease in myofibrillar Ca^{2+} sensitivity played no role. Acidification did not reduce force production or fatigue tolerance of human fibres. **Study IV** investigated the tentative role of SMase in disease-related muscle weakness and atrophy. SMase produced a prompt force decline by a reduction in SR Ca^{2+} release and decreased myofibrillar Ca^{2+} sensitivity, while promoting atrophy processes. Intramuscular SMase activity was elevated in heart failure compared to control individuals, and positively correlated with circulating markers of inflammation and atrophy, and with factors carrying prognostic value.

This thesis reveals differences in the intracellular Ca^{2+}-handling properties of myotubes and muscle fibres, and in the underlying causes of fatigue in humans and animals. This warrants caution when transferring findings in animal fibres and human myotubes to the situation in human skeletal muscle. The effects of vitamin D on muscle stem cells suggest an importance of vitamin D in skeletal muscle health. This should be considered in conditions of severe vitamin D deficiency, e.g. chronic kidney disease. Results implicate elevated SMase activity in the advent of muscle weakness and atrophy, suggesting SMase as a tentative target for therapeutic interventions in diseases associated with a loss of skeletal muscle function and mass.

List of scientific papers

This thesis is based on the following papers, herein referred to by their Roman numerals.

I. **Olsson K**, Cheng AJ, Alam S, Al-Ameri M, Rullman E, Westerblad H, Lanner JT, Bruton JD, Gustafsson T. Intracellular Ca^{2+}-handling differs markedly between intact human muscle fibres and myotubes. *Skeletal Muscle*. 2015;5:26. DOI 10.1186/s13395-015-0050-x

II. **Olsson K**, Saini A, Strömberg A, Alam S, Lilja M, Rullman E, Gustafsson T. Evidence for vitamin D receptor expression and direct effects of $1\alpha,25(OH)_2D_3$ in human skeletal muscle precursor cells. *Endocrinology*. 2016;157(1):98-111.

III. **Olsson K**, Cheng AJ, Al-Ameri M, Wyckelsma VL, Rullman E, Westerblad H, Lanner JT, Gustafsson T, Bruton JD. Impaired sarcoplasmic reticulum Ca^{2+} release is the major cause of fatigue-induced force loss in intact single fibres from human intercostal muscle. *The Journal of Physiology*. 2020;598(4):773–787.

IV. **Olsson K**, Cheng AJ, Al-Ameri M, Tardif N, Melin M, Rooyackers O, Lanner JT, Westerblad H, Gustafsson T, Bruton JD, Rullman E. Elevated sphingomyelinase activity in heart failure patients depresses skeletal muscle fibre force production and promotes atrophy. *Manuscript*.

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Karl Olsson
August 2020

Contents

1 Introduction and background

Several human diseases are associated with a high incidence of a profound and debilitating loss of skeletal muscle function and mass that result in a dismal life quality and poor prognosis (Anker *et al*., 1997; Hulsmann *et al*., 2004; Sokka *et al*., 2008; Chung *et al*., 2014; Powers *et al*., 2016). More than just the result of muscle disuse, disease-related changes inside the skeletal muscle fibres contribute to the progressive skeletal muscle dysfunction (Ward *et al*., 2003; Powers *et al*., 2016; Steinz *et al*., 2019). Experimental evidence from animal models has implicated dysfunctional intracellular Ca^{2+}-handling with the impaired muscle function (Reiken *et al*., 2003; Ward *et al*., 2003; Yamada *et al*., 2015b; Llano-Diez *et al*., 2016). Factors suggested to contribute to the changes in intracellular Ca^{2+}-handling include increased neuroendocrine activation, elevated circulating levels of pro-inflammatory cytokines, and altered sphingolipid metabolism (Reiken *et al*., 2003; Andersson & Marks, 2010; Nikolova-Karakashian & Reid, 2011; Reid & Moylan, 2011; Stasko *et al*., 2013). Meanwhile, vitamin D deficiency is a common finding in diseased individuals associated with muscle weakness and atrophy, ostensibly via both Ca^{2+}-dependent and Ca^{2+}-independent processes (Girgis *et al*., 2013). The current thesis investigated the intracellular mechanisms of human muscle fatigue and disease-related loss of muscle function and mass. A special focus was put on the role of altered intracellular Ca^{2+}-handling in these processes.

Skeletal muscle force development

Skeletal muscle contraction occurs when the contractile proteins myosin and actin in the force generating units, the sarcomeres, interact to form cycling cross bridges. In this reaction, ATP serves as an energy-carrying compound while being converted to ADP and free organic phosphate (P_i). In the resting muscle fibre, the myofilament regulatory protein tropomyosin forms a complex together with troponin C, T and I that blocks the myosin binding-sites on actin. A conformational change in tropomyosin, instigated by Ca^{2+} binding to troponin C, enables myosin and actin interaction. Elevations in the myoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) promote Ca^{2+} binding to troponin C, while Ca^{2+} dissociation from troponin C is promoted as $[Ca^{2+}]_i$ is lowered to its resting value (Gordon *et al*., 2000). Intracellular Ca^{2+} thus serves as the cellular signalling molecule that dictates initiation and termination of muscle contraction.

The level of $[Ca^{2+}]_i$ determines the number of cross bridges that can be formed and thus regulates the amount of force development within individual skeletal muscle fibres (Gordon *et al*., 2000). In this way, increasing levels of $[Ca^{2+}]_i$ results in larger force developments until all cross bridges are activated and the maximal Ca^{2+}-activated force is produced. There is a strong correlation between muscle fibre diameter and potential force development (Schantz *et al*., 1983). Muscle fibre diameter is determined by the balance between protein synthesis and degradation, but may also be modulated by the activity of muscle stem cells, as will be discussed further in the following sections.

Skeletal muscle excitation-contraction coupling

The events from sarcolemmal depolarisation to initiation of cross bridge cycling are described as excitation-contraction coupling. Depolarisation of the sarcolemma is initiated at the neuromuscular junction when acetylcholine is released by the α-motoneuron (Rebbeck *et al*., 2014). Acetylcholine binds to receptors at the sarcolemma triggering the opening of ion channels that allow passage of both Na^+ and K^+. This results in a localized depolarisation of the sarcolemma, that when it reaches a threshold value opens voltage-gated Na^+ channels (Nav1.4) generating an action potential that is propagated along the fibre via the sarcolemma and transverse tubular system (Gordon *et al*., 2000). In the transverse tubules, L-type voltage-activated Ca^{2+} channels (dihydropyridine receptor; DHPR) are activated by the propagating action potential. In skeletal muscle, DHPR mechanically activates the sarcoplasmic reticulum (SR) Ca^{2+} channels (type 1 ryanodine receptor; RyR1) (Tanabe *et al*., 1990; Rebbeck *et al*., 2014). At rest, the SR lumen contains 10-20 mM Ca^{2+}, the majority of which is bound to calsequestrin, while $[Ca^{2+}]_i$ is only about 50 nM. This large concentration gradient allows rapid elevation of $[Ca^{2+}]_i$ following RyR1 activation and opening (Allen *et al*., 2008).

When α-motoneuron activation ceases the sarcolemma is repolarized, RyR1 is inactivated, and SR Ca^{2+} release stops. The re-uptake of Ca^{2+} into the SR by the SR Ca^{2+}-ATPase (SERCA) returns $[Ca^{2+}]_i$ to resting levels. Ca^{2+} dissociates from troponin C causing tropomyosin to again block the myosin binding-sites on actin whereby cross bridge cycling terminates (Gordon *et al*., 2000).

Intracellular Ca^{2+} in skeletal muscle physiology and pathology

Intracellular Ca^{2+}-handling refers to the cellular regulation of $[Ca^{2+}]_i$ homeostasis, including the regulation of basal levels and fluctuations in $[Ca^{2+}]_i$, and the cellular response to fluctuations in $[Ca^{2+}]_i$. Intracellular Ca^{2+}-handling involves a large number of intracellular membrane or organelle bound proteins, as well as soluble cytosolic or organelle-confound proteins (Berchtold *et al*., 2000). In skeletal muscle, intracellular Ca^{2+}-handling is a key regulator and intracellular signalling molecule implicated in muscle force generation, fatigue development, regulation of gene expression and cellular metabolism (Berchtold *et al*., 2000; Lanner *et al*., 2006; Gundersen, 2011; Tavi & Westerblad, 2011).

Changes in $[Ca^{2+}]_i$ transient amplitude and duration, as well as frequency of the $[Ca^{2+}]_i$ transients, modulate the intracellular response to fluctuations in $[Ca^{2+}]_i$ (Dolmetsch *et al*., 1997; Tavi & Westerblad, 2011). Based on studies in animal and cell models, changes in baseline $[Ca^{2+}]_i$ have been implicated in both the physiological adaptation to exercise (e.g. mitochondrial biogenesis) (Bruton *et al*., 2010; Place *et al*., 2015) and maladaptation in disease (e.g. muscle weakness, fatigue and atrophy) (Bhattacharyya *et al*., 1993; Menconi *et al*., 2004; Smith & Dodd, 2007; Dridi *et al*., 2020; Kushnir *et al*., 2020).

Transient and prolonged changes in skeletal muscle intracellular Ca^{2+}-handling may be induced by several physiological and pathological stimuli. These include stimuli such as acute exercise and training, muscle disuse, hormonal and inflammatory factors (Berchtold *et al*., 2000; Allen *et al*., 2016). Transient changes (seconds to hours) in intracellular Ca^{2+}-handling are generally the result of changes in the levels of ions and metabolites that regulate the activity of Ca^{2+}-handling proteins. Prolonged changes (hours to days) may be caused by posttranslational modifications, changes in protein expression or stability of Ca^{2+}-handling proteins, or changes in the binding of regulatory proteins to Ca^{2+}-handling proteins (Berchtold *et al*., 2000; Place *et al*., 2015).

Skeletal muscle fatigue

Skeletal muscle fatigue has been defined as the reversible reduction in maximal voluntary force/power-producing capacity following prolonged or repeated contractions (Gandevia, 2001; Place *et al*., 2010). A distinction between central and peripheral components limiting force production in fatigue is often made. Central fatigue refers to processes within the nervous system whereas peripheral fatigue refers to processes at, or peripheral to, the neuromuscular junction (Taylor *et al*., 2006). It stands clear that both components participate to development of

fatigue, but the relative contribution of the two differs depending on the duration and frequency of contractions, duty cycle and external milieu (Gandevia, 2001). It is generally recognized that in the majority of situations a large part of fatigue development stems from peripheral components (Allen *et al*., 2008).

Cellular mechanisms of skeletal muscle fatigue

Studies in isolated intact and skinned mammalian muscle fibres have demonstrated three changes to ultimately cause the fatigue-induced force loss. These are (*i*) a reduction in myofibrillar maximal Ca^{2+}-activated force, (*ii*) decreased myofibrillar Ca^{2+} sensitivity, and (*iii*) reduced SR Ca^{2+} release (Westerblad & Allen, 1991, 1993a; Allen *et al*., 2008) (**Figure 1.1**). The relative importance of these changes differs during three distinctly identifiable phases during fatigue; an initial phase of depressed force while tetanic $[Ca^{2+}]_i$ increases, explained by a reduced myofibrillar maximal Ca^{2+}-activated force; a second phase where tetanic $[Ca^{2+}]_i$ and force is little affected; followed by a final phase in which both tetanic $[Ca^{2+}]_i$ and force rapidly declines. This third phase is explained by a concurrent reduction in SR Ca^{2+} release and decreased myofibrillar Ca^{2+} sensitivity (Westerblad & Allen, 1991, 1993a; Allen *et al*., 2008).

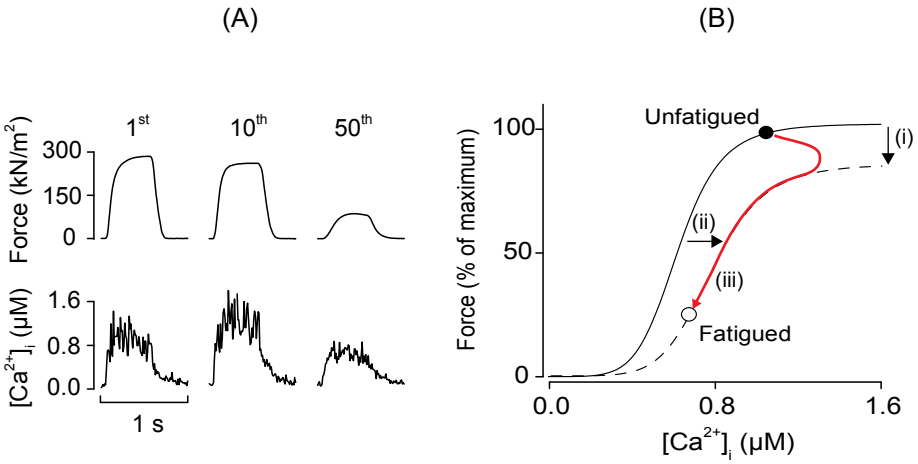

Figure 1.1. **Illustration of the cellular mechanisms of fatigue in rodent muscle fibres.** *A*, force and $[Ca^{2+}]_i$ in the 1st, 10th and last (50th) tetanus of fatigue. Note the initial increase in tetanic $[Ca^{2+}]_i$ while force decreases between the 1st and 10th tetanus. *B*, proposed sequence of events during fatigue development (red line) and mechanisms causing fatigue in rodent muscle fibres: (*i*) a reduction in myofibrillar maximal Ca^{2+}-activated force, (*ii*) decreased myofibrillar Ca^{2+} sensitivity, and (*iii*) reduced tetanic $[Ca^{2+}]_i$ due to impaired SR Ca^{2+} release. Force-$[Ca^{2+}]_i$ relation in unfatigued (solid line) and fatigued (dashed line) fibres. Redrawn from Allen, Lamb & Westerblad; J Appl Physiol 104: 296–305, 2008.

Accumulation of P_i is believed to be of fundamental importance to the intrinsic skeletal muscle changes that ultimately causes the force depression during fatigue. The initial decline in myofibrillar maximal Ca^{2+}-activated force is not seen in mouse muscle fibres deficient of creatine kinase where there is little change in P_i (Dahlstedt *et al*., 2000), and P_i depresses tetanic force in skinned fibres (Pate & Cooke, 1989; Millar & Homsher, 1990). The reduction in tetanic $[Ca^{2+}]_i$ is believed to mainly stem from perturbed SR Ca^{2+} release resulting from Ca^{2+}-P_i precipitation in the SR (Fryer *et al*., 1995; Westerblad & Allen, 1996), and the combined effects of reduced cytosolic [ATP] and increased cytosolic free $[Mg^{2+}]$, which inhibits the RyR1 channel (Blazev & Lamb, 1999). Accordingly, the fall in tetanic $[Ca^{2+}]_i$ is greatly delayed when creatine kinase is inhibited in rodent fibres (Dahlstedt & Westerblad, 2001), and injection of P_i into single intact mouse fibres causes a marked fall in SR Ca^{2+} release and tetanic force (Westerblad & Allen, 1996). Reduced myofibrillar Ca^{2+} sensitivity is caused by an accumulation of P_i and a reduction in intracellular pH (pH_i), but may also be modulated by reactive oxygen and nitrogen species (RONS) (Dahlstedt *et al*., 2001; Allen *et al*., 2008; Lamb & Westerblad, 2011).

Intramuscular acidosis and skeletal muscle fatigue development

In exercising humans, pH_i has been reported to fall ~0.5 pH units following intense muscle activity (Sahlin *et al*., 1975; Sahlin *et al*., 1976; Bangsbo *et al*., 1996). Historically, intramuscular acidosis has been acclaimed a major role in the force decline during fatigue (Fitts, 2016). The underlying observations supporting this view include observations from exercising humans and studies from isolated animal muscle fibres.

Initial studies found a good temporal correlation between intramuscular acidification and fatigue (Dawson *et al*., 1978). In coherence with this finding, a reduction in maximal Ca^{2+}-activated force was demonstrated upon acidification of isolated animal muscles (Donaldson *et al*., 1978; Fabiato & Fabiato, 1978). This notion was later challenged as the force-depressive effect of acidification was found to be highly temperature-dependent and to be blunted when studied at physiological temperatures (Pate *et al*., 1995; Westerblad *et al*., 1997). Notably, the fatigue tolerance of isolated rodent fibres fatigued under acidic conditions was unaltered when studied at near-physiological temperatures (Bruton *et al*., 1998).

Recovery from fatigue

Recovery of force producing capacity following fatigue follows a time course that is largely dependent on the metabolic properties of the muscle fibre. While oxidative slow-twitch fibres recover their force producing capacity nearly instantly, fast-twitch fibres recover more slowly (Allen *et al*., 2008). In fast-twitch

fibres it is possible to discern a difference in the time course for recovery depending on the frequency of stimulation used to initiate contraction. At high-frequency stimulation (80-100 Hz) recovery from fatigue occurs within minutes, while at low-frequency stimulation (20-30 Hz) recovery is not complete until several hours (Edwards *et al*., 1977; Bruton *et al*., 2008). This delayed force recovery at low frequencies has been termed prolonged low-frequency force depression (PLFFD).

Theoretically, PLFFD could be caused by an impaired SR Ca^{2+} release and/or reduced myofibrillar Ca^{2+} sensitivity. This is explained by the non-linear force-$[Ca^{2+}]_i$ relation, which is flat at high- while steep at low-stimulation frequencies. Hence, a reduction in tetanic $[Ca^{2+}]_i$ or a decrease in myofibrillar Ca^{2+} sensitivity will result in a more marked force reduction at low- than at high-frequencies (Westerblad et al., 1993; Bruton et al., 2008; Cheng et al., 2015). The relative contribution of the two underlying causes of PLFFD varies with recovery time (Watanabe et al., 2015; Watanabe & Wada, 2016) and experimental species (Bruton et al., 2008).

Models to study skeletal muscle Ca^{2+}-handling, force and fatigue

Attempts to address the mechanisms behind skeletal muscle fatigue have employed experimental models that range from exercising humans to isolated single myofibrils (Lamb, 2002; Allen *et al*., 2008). Given the central role of intracellular Ca^{2+} in the physiological events responsible for skeletal muscle force generation and fatigue development, experimental models that enable continuous recordings of $[Ca^{2+}]_i$ and force are best suited for such mechanistic studies. At the time this thesis work commenced, intact single fibres isolated from animal muscles were the only model to entirely fulfil these requirements (Place *et al*., 2010). Due to the possibility of species differences, it was unknown to what extent findings in animal models would translate to the human setting. Alternative experimental models enabling studies of human skeletal muscle cells included *in vivo* studies, skinned skeletal muscle fibres, and cultures of myoblasts and myotubes. However, as will be alluded to next, inherent limitations of these models have limited their applicability in mechanistic studies of intracellular Ca^{2+}-handling and force production.

Human skeletal muscle *in vivo*

The intact human skeletal muscle *in vivo* with maintained blood supply, neural innervation of motor units and, often, mixture of fibre types, extracellular matrix and tendinous attachments intact is arguably the gold standard for experiments on human muscle functions. In the early 1950's, non-invasive experimental protocols

were developed to study human fatigue by analysing the reduction in maximal voluntary force following isometric contractions (Merton, 1954). Later, the possibility of EMG and percutaneous stimulation of the motor nerve or the muscle itself enabled the differentiation of peripheral and central components of human muscle fatigue (Bigland-Ritchie *et al*., 1978). The muscle biopsy technique (Bergström, 1962), and non-invasive nuclear magnetic resonance spectroscopy (Sapega *et al*., 1987), made it possible to study the correlation of skeletal muscle metabolic and ionic changes with fatigue development. Despite these advances, it remains difficult to distinguish between the different factors that may contribute to fatigue in human skeletal muscle *in vivo* experiments (Allen *et al*., 2008). For these types of mechanistic studies, direct assessments of changes in $[Ca^{2+}]_i$ in parallel with measurements of force are genereally considered necessary. Techniques that enable this type of studies in humans *in vivo* are yet to be developed.

Isolated intact single skeletal muscle fibres

Intact single skeletal muscle fibres can be isolated from animal muscles by either enzymatic dissociation or manual dissections. While the former represents a relatively straightforward procedure to obtain several muscle fibres per muscle bundle, the latter is a tedious dissection using forceps, micro-scissors and microscopes to obtain a single intact muscle fibre (Cheng & Westerblad, 2017). The great advantage of the latter approach is that, while the tendons are lost during enzymatic dissociation, they remain intact following manual dissection. This allows mounting of the muscle fibre in a force transducer making force recordings possible. Fluorescent Ca^{2+} indicator dyes can be introduced into viable muscle fibres, which enable continuous measurements of force and $[Ca^{2+}]_i$ in living muscle fibres (Place *et al*., 2010; Cheng & Westerblad, 2017).

Skinned skeletal muscle fibres

Skinned skeletal muscle fibres are muscle fibres in which the sarcolemma has been disrupted or removed. This can be achieved either by chemical permeabilisation of the sarcolemma or by mechanically removing the sarcolemma (Lamb, 2002). The major advantage of the skinned muscle fibre model is that it does not require that intact muscle fibres be collected, which can be difficult to nearly impossible depending on the size of the muscle (Cheng & Westerblad, 2017). Notably, skinned muscle fibres can be obtained from, e.g., percutaneous muscle biopsies, enabling studies in human skeletal muscle (Place *et al*., 2010).

Limitations of this model include a loss of soluble cytosolic proteins and the naturally occurring build-up of metabolic by-products that may be of importance in fatigue development. Skinned fibre experiments are generally conducted at temperatures lower than those prevailing *in vivo* and, as alluded to earlier, this may be problematic when considering the mechanistic aspects of fatigue (Lamb, 2002; Allen *et al*., 2008). In addition, although feasible in the less used model of

mechanically skinned fibres (Posterino *et al*., 2000), the standard procedure of chemically skinned fibres does not allow for analysis of SR Ca^{2+} release and excitation-contraction coupling (Lamb, 2002; Allen *et al*., 2008).

Myoblasts and myotubes

Satellite cells are skeletal muscle resident stem cells involved in the maintenance and adaptive processes of skeletal muscle tissues (Mauro, 1961; Relaix & Zammit, 2012). Satellite cells can be extracted from human muscle biopsies, activated into proliferating myoblasts and differentiated into multinuclear myotubes *ex vivo* (Blau & Webster, 1981). Upon differentiation of myoblasts into myotubes, several morphological, metabolic and biochemical features similar to those in adult skeletal muscle fibres have been reported (Berggren *et al*., 2007; Aas *et al*., 2013). This together with the advantage that myotubes retain donor-specific traits have rendered myotubes a widely used *ex vivo* model of human skeletal muscle (Peter *et al*., 2009; Ullrich *et al*., 2011; Nikolic *et al*., 2012; Aas *et al*., 2013; Scheler *et al*., 2013). However, differences in metabolic (Sarabia *et al*., 1992; Baker *et al*., 2003), contractile and intracellular Ca^{2+}-handling properties (Tanaka *et al*., 2000; Bandi *et al*., 2008; Rokach *et al*., 2013; Cheng *et al*., 2014; Smith *et al*., 2014; Madden *et al*., 2015) have been reported between myotubes and muscle fibres. These limitations cast doubt as to whether findings in myotubes are transferrable to the adult muscle fibre.

Intrinsic skeletal muscle changes in disease

Disease-related changes affecting skeletal muscle force producing capacity and fatigue resistance may occur at any level from the brain to the contractile proteins. Although likely the result of a combination of factors, it is clear that changes intrinsic to the skeletal muscle contributes to the loss of skeletal muscle function in patients suffering from chronic diseases including heart failure and rheumatoid arthritis (Middlekauff, 2010; Yamada *et al*., 2017; Steinz *et al*., 2019), but also in patients afflicted by acute critical illness myopathy (Friedrich *et al*., 2015).

In heart failure patients, this is demonstrated by the finding of a remaining muscle force deficit when force is normalized to muscle cross sectional area, leg muscle mass, or body weight (Lipkin *et al*., 1988; Harrington *et al*., 1997; Toth *et al*., 2006; Toth *et al*., 2010; Toth *et al*., 2012). A similar qualitative decline in muscle function is found in rheumatoid arthritis and critical illness myopathy patients (Helliwell & Jackson, 1994; Friedrich *et al*., 2015), and rodent disease models (Yamada *et al*., 2009; Yamada *et al*., 2015a; Yamada *et al*., 2015b; Llano-Diez *et al*., 2016; Steinz *et al*., 2019).

Intracellular Ca^{2+}-handling and disease-related muscle dysfunction

Altered intracellular Ca^{2+}-handling has been implicated in disease-related quantitative and qualitative changes in skeletal muscle. Changes in intracellular Ca^{2+}-handling may involve principally two steps in the excitation-contraction coupling process that would directly affect muscle contractile function and resistance to fatigue: (*i*) the release/reuptake of SR Ca^{2+} and (*ii*) the myofibrillar Ca^{2+} sensitivity. Additionally, changes in basal $[Ca^{2+}]_i$ or $[Ca^{2+}]_i$ transient amplitude and/or duration regulate, e.g., muscle protein metabolism and the amount of contractile proteins available for force production (Bhattacharyya *et al*., 1993; Menconi *et al*., 2004; Smith & Dodd, 2007; Dridi *et al*., 2020; Kushnir *et al*., 2020).

A reduction in SR Ca^{2+} release and decreased myofibrillar Ca^{2+} sensitivity has been found to contribute to the muscle weakness observed in animal models of heart failure (Perreault *et al*., 1993; Reiken *et al*., 2003; Ward *et al*., 2003), rheumatoid arthritis (Yamada *et al*., 2009; Yamada *et al*., 2015b), critical illness myopathy (Callahan *et al*., 2001; Llano-Diez *et al*., 2016), and normal ageing (Andersson *et al*., 2011). Due to technical difficulties, mechanistic studies of this sort in humans are scarce. However, perturbed action potential-evoked Ca^{2+} transient amplitude and release in isolated skeletal muscle fibre segments from heart failure patients have been reported (DiFranco *et al*., 2014). Additionally, the quantity and extent of posttranslational modifications of intracellular Ca^{2+}-handling proteins differ between human heart failure patients and controls, indicating plausible differences in functional Ca^{2+}-handling properties (Middlekauff *et al*., 2012; Rullman *et al*., 2013) in this patient group.

Causes of disease-related changes in intracellular Ca^{2+}-handling

Studies in animal models have demonstrated that a loss of skeletal muscle mass and function, related to altered intracellular Ca^{2+}-handling, is manifested in several diseases of otherwise different underlying aetiology (Reiken *et al*., 2003; Yamada *et al*., 2015b; Llano-Diez *et al*., 2016). It is possible that factors not related to the disease process in itself, e.g., muscle disuse, or processes unique for each disease may explain these common manifestations. An alternative hypothesis is that the inherent biological response to several diseases shares common features, and that these contribute to the impaired muscle function. In support for this, several of these diseases exhibit common features, such as an increased neuroendocrine activation, elevated circulating levels of pro-

inflammatory cytokines, and altered sphingolipid metabolism (Andersson & Marks, 2010; Nikolova-Karakashian & Reid, 2011; Reid & Moylan, 2011). In animal models, these have all been demonstrated to induce changes in skeletal muscle intracellular Ca^{2+}-handling and to impair muscle function.

Neuroendocrine activation

Posttranslational modifications of RyR1, including phosphorylation, oxidation and nitrosylation, have been demonstrated in human heart failure patients (Rullman *et al*., 2013). In animal models, these modifications have been shown to result in dissociation of the stabilising protein FKBP12 from RyR1, increasing the leakage of SR Ca^{2+} into the cytosol. This is turn causes an elevation in baseline $[Ca^{2+}]_i$ and a depletion of SR Ca^{2+}, ultimately causing a reduction in tetanic $[Ca^{2+}]_i$ amplitude and subsequent force decline (Ward *et al*., 2003). Excessive neuroendocrine activation in heart failure has been suggested to cause skeletal muscle weakness and fatigue intolerance due to hyperphosphorylation of RyR1 via β-adrenergic activation (Reiken *et al*., 2003).

Cytokines

A number of circulating proinflammatory cytokines are elevated in heart failure, rheumatoid arthritis and critical illness myopathy. Of these, TNFα is the cytokine most widely recognized to correlate with disease severity and poor prognosis (Rauchhaus *et al*., 2000; Conraads *et al*., 2003; Friedrich *et al*., 2015). Albeit other circulating cytokines are elevated in these conditions, the correlation of these cytokines to muscle dysfunction and prognosis is weaker and more ambiguous (Testa *et al*., 1996; Friedrich *et al*., 2015). Increased levels of circulating TNFα, or soluble forms of TNF receptor I or II, which are believed to reflect an increased activity of TNFα, are elevated in heart failure (Levine *et al*., 1990; Torre-Amione *et al*., 1996; Rauchhaus *et al*., 2000), and especially so in the most symptomatic heart failure patients (i.e. NYHA class III-IV) (Ferrari *et al*., 1995; Testa *et al*., 1996; Torre-Amione *et al*., 1996; Rauchhaus *et al*., 2000; Deswal *et al*., 2001), and in cachectic patients (Levine *et al*., 1990).

Exogenously administered TNFα depresses cardiomyocyte force by reducing tetanic $[Ca^{2+}]_i$ (Yokoyama *et al*., 1993). In skeletal muscle, TNFα has been demonstrated to reduce membrane excitability suggesting that it may inactivate DHPR and thus reduce tetanic $[Ca^{2+}]_i$ and force (Tracey *et al*., 1986). Later studies in isolated rodent skeletal muscle fibres have shown that TNFα acutely reduces tetanic force without affecting tetanic $[Ca^{2+}]_i$ (Reid *et al*., 2002), by a RONS-dependent mechanism (Reid *et al*., 2002; Stasko *et al*., 2013). Notably, changes in intracellular Ca^{2+}-handling via RONS-induced modifications of the RyR1 have been suggested in human heart failure patients (Rullman *et al*., 2013).

Sphingolipid metabolism and sphingomyelinase activity

The sphingomyelinase family is a group of biochemically and genetically different enzymes, all of which hydrolyse sphingomyelin to ceramide and phosphorylcholine (Nikolova-Karakashian & Reid, 2011). Circulating sphingomyelinase activity is elevated in human heart failure (Doehner *et al*., 2007), rheumatoid arthritis (Hanaoka *et al*., 2018) and critical illness myopathy patients (Claus *et al*., 2005). In heart failure patients, circulating sphingomyelinase activity is positively correlated to both circulating TNFα and TNF-RI while negatively correlated to muscle strength measures and lean tissue mass (Doehner *et al*., 2007). Moreover, circulating sphingomyelinase activity is increased in a stepwise fashion according to NYHA class while related to impaired survival, independent of age, NYHA class or mean blood pressure (Doehner *et al*., 2007). Similarly, an elevation of sphingomyelinase activity predicts a worse outcome in patients treated at the intensive care unit, while inhibition of sphingomyelinase activity has been found to increase survival in a mouse model of endotoxic shock (Claus *et al*., 2005).

In rodent skeletal muscle, exogenous sphingomyelinase increases ceramides and oxidant activity while perturbing force production (Ferreira *et al*., 2010; Ferreira *et al*., 2012; Loehr *et al*., 2014; Bost *et al*., 2015), and fatigue resistance (Ferreira *et al*., 2010; Ferreira *et al*., 2012; Loehr *et al*., 2014). Sphingomyelinase-induced attenuation of force has been suggested to result from changes in maximum Ca^{2+}-activated force and a reduction in myofibrillar Ca^{2+} sensitivity (Ferreira *et al*., 2012). The current hypothesis is that increased levels of pro-inflammatory cytokines and RONS contribute to perturbed muscle function in diseases characterized by elevated sphingomyelinase activity via sphingomyelinase-dependent effects on skeletal muscle force producing capacity and fatigue resistance (Marathe *et al*., 1998; Wong *et al*., 2000; Jenkins *et al*., 2010; Nikolova-Karakashian & Reid, 2011; Moylan *et al*., 2014).

Disease-related loss of muscle mass

Disease-related loss of muscle mass results in a dismal life quality and has been reported as an independent risk factor for mortality (Anker *et al*., 1997; Hulsmann *et al*., 2004; Sokka *et al*., 2008; Chung *et al*., 2014; Powers *et al*., 2016). The net protein balance, i.e. the ratio of protein synthesis and degradation, dictates muscle fibre size (Phillips *et al*., 2012). When muscle protein degradation exceeds synthesis, muscle protein depletion ensues and the muscle fibre atrophies. At the whole tissue level, muscle atrophy may also be caused by the loss of individual muscle fibres (Wilkinson *et al*., 2018). In several diseases, both a negative protein balance and a loss of muscle fibres have been shown to contribute to the loss of muscle mass (Powers *et al*., 2016). Disease-related modifications of Ca^{2+}-

regulated process have been postulated to trigger these changes (Bhattacharyya *et al*., 1993; Menconi *et al*., 2004; Smith & Dodd, 2007; Dridi *et al*., 2020; Kushnir *et al*., 2020).

A third potential cause of disease-related muscle atrophy is a reduced hypertrophic response and regenerative capacity of the skeletal muscle due to perturbed muscle stem cell function and abundance (McKenna & Fry, 2017; Wilkinson *et al*., 2018). Muscle stem cell functions are regulated by local and systemic factors that modulate their proliferation and differentiation. One such factor that has increasingly been linked to skeletal muscle health and intimately linked to the regulation of muscle stem cell functions is vitamin D (Girgis *et al*., 2013; Girgis *et al*., 2014a).

The role of vitamin D in skeletal muscle physiology and pathology

Deficiency of Vitamin D is a widely spread and highly prevalent condition, and has been associated with skeletal muscle weakness and atrophy (Girgis *et al*., 2013). The major circulating form of vitamin D consists of 25(OH)D, which is believed to be biologically inactive. 25(OH)D is hydroxylated by the enzyme 1α-hydroxylase, present in the proximal tubules of the kidney, to form the biologically active 1α,25(OH)$_2$D. The half-life of 1α,25(OH)2D is short, and the synthesis strictly controlled so that serum levels are usually well maintained despite very low levels of 25(OH)D (Need *et al*., 2008). Assessment of vitamin D status is therefore made by measuring the serum concentration of 25(OH)D, upon which the diagnosis of vitamin D deficiency is made (Hollis & Wagner, 2005).

The vitamin D receptor has been reported to be present in virtually all cells of the body, including some reports claiming its presence in human skeletal muscle fibres (Pojednic *et al*., 2015). Proposed effects of vitamin D in skeletal muscle include an increase in force generation via non-genomic actions on intracellular Ca^{2+}-handling, and an anabolic response via transcriptional regulation (Girgis *et al*., 2013; Bass *et al*., 2020). In rodent muscles, overexpression of the vitamin D receptor induces a hypertrophic response by stimulating protein synthesis, and possibly muscle stem cell number and activity (Bass *et al*., 2020). Notably, vitamin D has been suggested to reduce the expression of myostatin, a well-established negative regulator of muscle mass (McPherron *et al*., 1997), in rodent skeletal muscle cells (Garcia *et al*., 2011; Girgis *et al*., 2014a). However, a direct effect of vitamin D on skeletal muscle cells has been a subject of controversy, with even the presence of the vitamin D receptor in the skeletal muscle tissue in question (Wang & DeLuca, 2011; Bouillon *et al*., 2014; Pike, 2014).

2 Objectives and outline of the thesis

The overarching aim of the current thesis was to study the intracellular mechanisms of human muscle fatigue and disease-related loss of muscle function and mass. A special focus was put on the tentative role of altered intracellular Ca^{2+}-handling in these processes. An integrative approach spanning from single intact muscle fibres, isolated muscle stem cells, to whole muscle biopsies from healthy and diseased individuals was employed.

The specific aims of the studies were as follows:

Study I To establish an experimental model suitable for mechanistic studies of intracellular Ca^{2+}-handling, force generation and fatigue in human skeletal muscle.

Study II To test the hypothesis that vitamin D plays a role in maintaining human skeletal muscle function and mass by regulating muscle stem cell functions.

Study III To investigate the cellular mechanisms of human muscle fatigue and the effects of acidosis on muscle performance.

Study IV To test the hypothesis that elevated sphingomyelinase activity is implicated in disease-related loss of muscle function and mass by affecting intracellular Ca^{2+}-handling and muscle atrophy processes.

3 Summary of study results

The following section provides a summary of the key results of studies I-IV. For detailed results the reader is referred to the individual studies presented in sections four to seven.

Study I: *Ex vivo* models of human skeletal muscle

In study I, we took advantage of the relatively short muscle fibres of the accessory respiratory muscles located between the ribs of humans, the intercostal muscles, to manually dissect intact single fibres. During thoracotomies performed for medical reasons, the intercostal muscles are exposed and dissected to access the thoracic cavity. Thus, they are accessible for muscle biopsies without unwarranted inconvenience or additional risk for the patient. By this approach we obtained intact single human muscle fibres that enabled electrically evoked contractions, while continuously measuring $[Ca^{2+}]_i$ and force. Human myoblasts, isolated from vastus lateralis muscle biopsies, were differentiated *ex vivo* to form myotubes, and their contractile function and Ca^{2+}-handling characteristics were compared to those of adult intercostal muscle fibres.

Upon differentiation, human myoblasts fused to form multinucleated myotubes with an increased expression of Ca^{2+}-handling proteins and functionality of the initial events of the excitation-contraction coupling cascade. Increased expression of contractile proteins and initiation of structural organisation were also evident upon differentiation. Yet, we found major remaining differences when compared to the adult muscle fibre: none of the myotubes contracted, and the Ca^{2+} transient decay upon electrical stimulation was markedly prolonged in myotubes (**Figure 3.1*A-B***). Subsequent analyses suggested that these remaining differences were due to dissimilarities in regards to the protein abundance and co-localisation of Ca^{2+}-handling proteins, and ultrastructure (**Figure 3.1*C-D***).

The duration of $[Ca^{2+}]_i$ transients have been demonstrated to affect the cellular signalling initiated by increased $[Ca^{2+}]_i$ (Berridge, 1990, 1992; Dolmetsch *et al*., 1997; Berridge *et al*., 2003; Clapham, 2007; Tavi & Westerblad, 2011). Thus, when studying Ca^{2+}-dependent processes such as gene expression and metabolism, myotubes are likely to provide different answers to those given by intact adult muscle fibres.

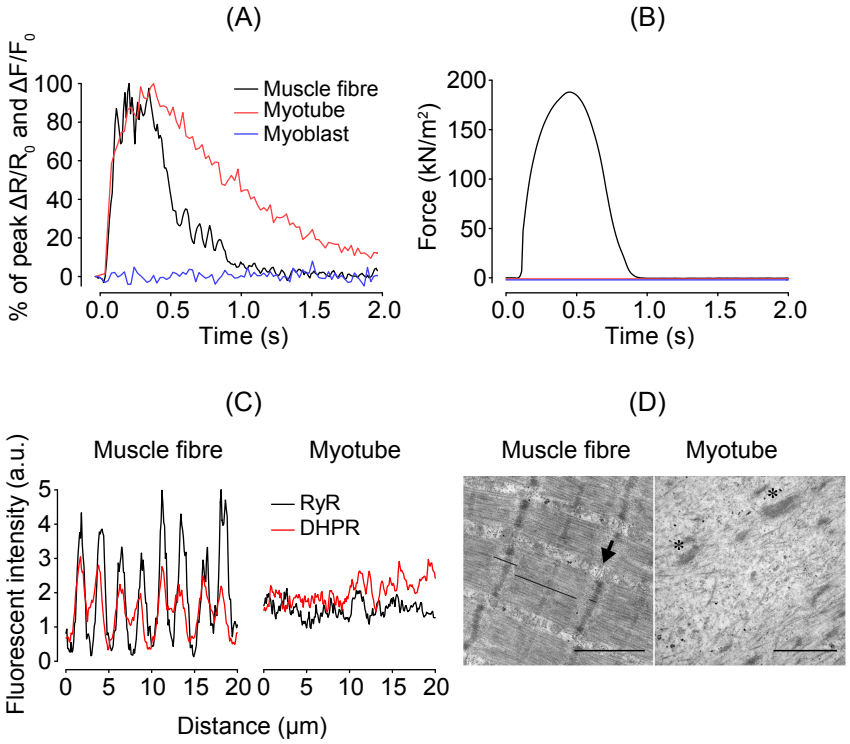

Figure 3.1. **Human muscle fibres and myotubes showed marked differences in Ca^{2+}-handling characteristics and contractile ability.** Changes in Ca^{2+} indicator dye intensity (*A*) and force (*B*) following electrical stimulation of a human muscle fibre, myotube and myoblast. Fluorescent intensity for RyR and DHPR (*C*), and TEM images of the ultrastructure (*D*) in a human muscle fibre and myotube. Note the characteristic arrangement of myofibrils separated by triads (arrow) at the junction of the A and I bands in fibres while only rudimentary myofilament structures (*) were seen in myotubes.

Study II: Effects of vitamin D in human skeletal muscle

Contemplating previous findings and the results in study I, we considered human myoblasts, myotubes and muscle fibres to represent distinct stages of muscle development. In study II, we took advantage of this to investigate the presence of components of the vitamin D endocrine system, including the vitamin D receptor, and the vitamin D metabolising enzymes CYP24A1 and CYP27B1, in human muscle cells at different developmental stages. The effects of vitamin D on the regulation of processes involved in muscle regeneration and growth were investigated in human myoblasts.

The vitamin D receptor was readily detectable in human myoblasts and myotubes, while non-detectable in human adult skeletal muscle (**Figure 3.2*A***). Attention was therefore focused on the effects of vitamin D on human myoblast proliferation and differentiation. Results revealed a profound inhibitory effect by the biologically active form of vitamin D ($1\alpha,25(OH)_2D$) on myoblast proliferation and differentiation. No effects by the biologically inactive form $25(OH)_2D$ were detected in human myoblasts (**Figure 3.2*B***).

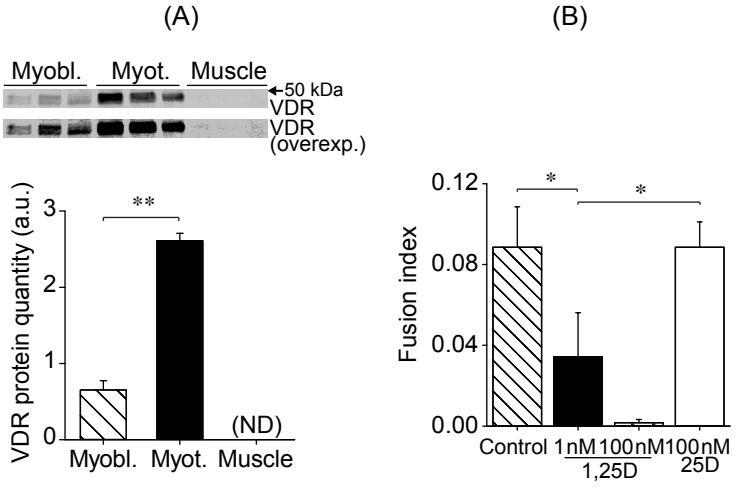

Figure 3.2. **The vitamin D receptor was readily detected in human muscle stem cells and vitamin D markedly inhibited human myotube formation.** *A*, protein quantity of the vitamin D receptor (VDR) in human primary myoblasts, myotubes and adult skeletal muscle. *B*, fusion index following 8 days of differentiation in the presence of 1 nM or 100 nM $1\alpha,25(OH)_2D_3$, or 100 nM $25(OH)D_3$. Data are presented as the mean ±SEM. *P<0.05, **P<0.01. (ND) = non-detectable.

Global gene expression analysis provided mechanistic insight by demonstrating an altered expression of cell cycle regulators consistent with a block of cell cycle progression at the G_1/S restriction point. Further, vitamin D reduced the expression of MyoD, myogenin and MEF2C, myogenic regulatory factors acknowledged for their role in muscle stem cell commitment and differentiation (Relaix & Zammit, 2012). Gene-ontology enrichment of down-regulated genes identified GO categories involved in 'muscle organ development'. Network analysis using IPA identified pathways annotated as 'differentiation of myoblasts' and 'differentiation of muscle cells' among the most highly inhibited biological functions. Altered expression of genes intimately involved in muscle stem cell self-renewal lead us to suggest a role of the biologically active form of vitamin D in the maintenance of the muscle stem cell pool (**Figure 3.3**).

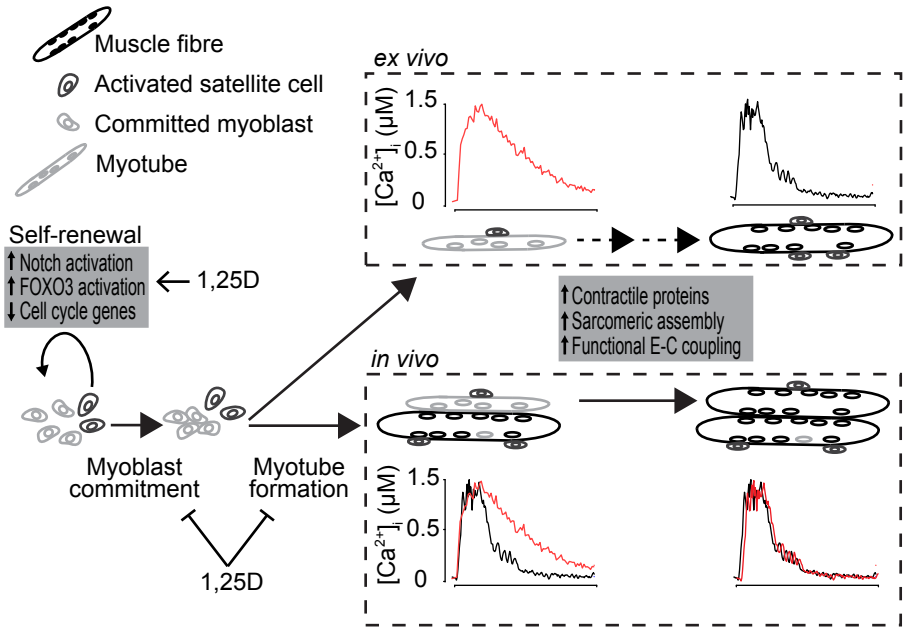

Figure 3.3. **Illustration of the results from studies I and II.** Vitamin D promotes satellite cell self-renewal while inhibiting myoblast differentiation. Activated myoblasts form myotubes both *in vivo* and *ex vivo*, but only *in vivo* do myotubes fully mature into muscle fibres with functional excitation-contraction (E-C) coupling and contractile ability.

Study III: Cellular mechanisms of human skeletal muscle fatigue

In study III, the cellular mechanisms of human muscle fatigue following near maximal isometric contractions were investigated by continuous measurements of force and $[Ca^{2+}]_i$ transients. Based on their ability to maintain force >40% of the unfatigued value during 500 tetani, two distinct groups of fibres were distinguished: fatigue sensitive and fatigue resistant.

In both groups of fibres, the fatigue-induced force loss was primarily caused by an impaired SR Ca^{2+} release, while the myofibrillar Ca^{2+} sensitivity was either unaltered (fatigue-sensitive fibres) or increased (fatigue-resistant fibres) (**Figure 3.4**). Integrating these results, fatigue development in human muscle fibres is different to that in rodent muscle fibres in that reduced myofibrillar Ca^{2+} sensitivity plays no role in the force loss in human fibres.

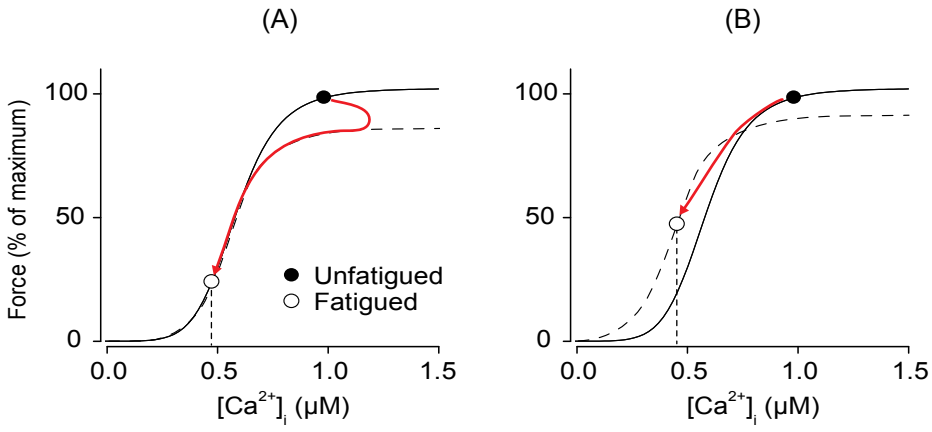

Figure 3.4. **Illustration of the cellular mechanisms of fatigue in human muscle fibres.** *A* and *B*, contractile-induced changes in tetanic force, $[Ca^{2+}]_i$ and force-$[Ca^{2+}]_i$ relationship in human fatigue-sensitive and fatigue-resistant muscle fibres, respectively. Note that in both human fatigue-sensitive and fatigue-resistant fibres the main cause of fatigue was a reduced SR Ca^{2+} release while myofibrillar Ca^{2+} sensitivity was either unaltered (fatigue-sensitive fibres) or increased (fatigue-resistant fibres) following fatigue.

In fatigue-resistant fibres, recovery was complete 15 minutes after cessation of fatiguing contractions. In contrast, in fatigue-sensitive fibres the force at low-frequency stimulation was markedly lower, while force at high-frequency stimulation was recovered, compared to the unfatigued value. This phenomenon, known as prolonged low-frequency force depression (PLFFD), was exclusively due to an impaired SR Ca^{2+} release. Myofibrillar Ca^{2+} sensitivity and maximal force producing capacity was unaltered (**Figure 3.5**). Cumulatively, these results demonstrate a fundamental importance of impaired SR Ca^{2+} release to the force depression following fatigue and in the early recovery period in human muscle fibres.

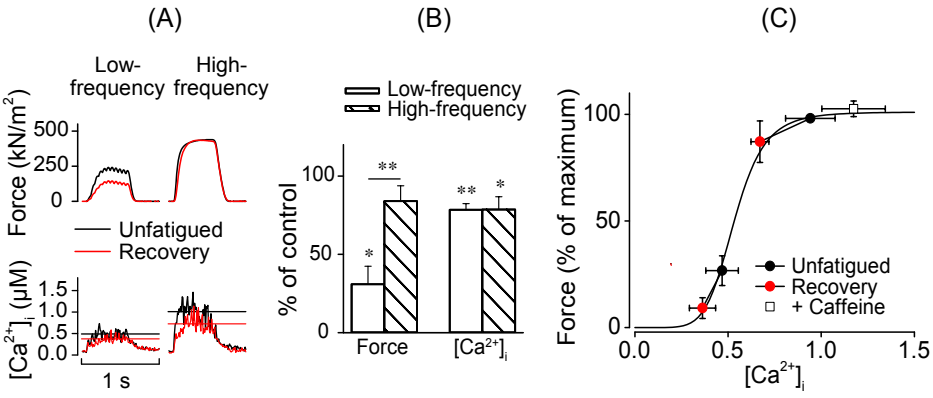

***Figure 3.5*. PLFFD in human muscle fibres was caused exclusively by an impaired SR Ca^{2+} release.** *A*, representative force and $[Ca^{2+}]_i$ records in a fatigue-sensitive muscle fibre at low- and high-frequency stimulation before (unfatigued) and 15 min after (recovery) fatiguing stimulation. *C*, PLFFD was evident in human fatigue-sensitive muscle fibres. *D*, the force decline in fatigue-sensitive fibres during recovery followed the unfatigued force-$[Ca^{2+}]_i$ relationship with force fully restored to unfatigued levels in the presence of 5 mM caffeine. Data are means ± SEM. * $P < 0.05$, ** $P < 0.01$.

Intracellular acidosis has historically been ascribed a major causative role in the fatigue-induced force loss following intense contractile activity (Fitts, 2016). However, as alluded to previously, this view has later been challenged by findings in animal isolated muscles studied at or near physiological temperatures (Pate *et al*., 1995; Westerblad *et al*., 1997; Bruton *et al*., 1998). We were therefore interested in the effects of intracellular acidosis on the force-$[Ca^{2+}]_i$ relation and fatigue resistance of human fibres at 37° C. In unfatigued human muscle fibres, intracellular acidosis reduced the myofibrillar Ca^{2+} sensitivity, but this was completely offset by an increase in tetanic $[Ca^{2+}]_i$ so that force was maintained (**Figure 3.6*A-B***). Results further suggested that the fatigue-resistance of human fibres was unaltered upon acidification (**Figure 3.6*C-D***).

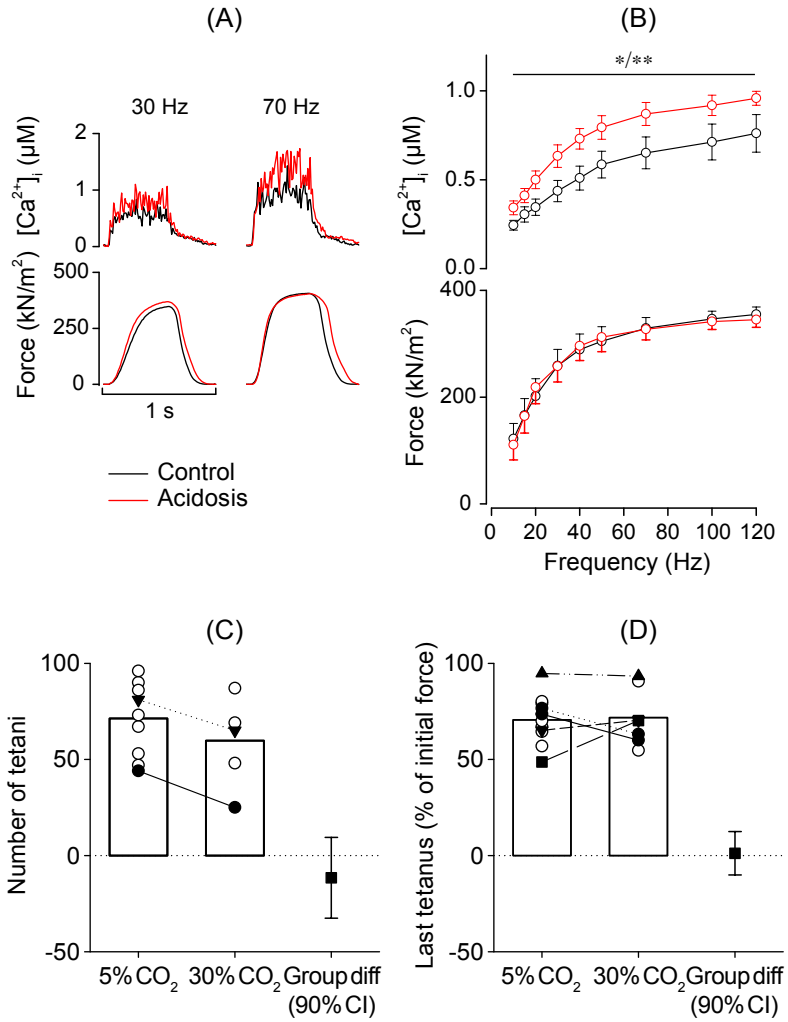

***Figure 3.6*. Acidosis did not reduce force production, nor decrease the fatigue tolerance of human intercostal muscle fibres.** *A*, representative $[Ca^{2+}]_i$ and force records in a human muscle fibre during a 30 Hz and 70 Hz tetanus under control (5% CO_2) and acidic (30% CO_2) conditions. *B*, acidification resulted in increased tetanic $[Ca^{2+}]_i$ while force production was unaltered. *C* and *D*, number of tetanic contractions required to reach 40% of the initial force in fatigue-sensitive (*C*) and fatigue-resistant (*D*) human fibres. Data are means with individual data points as well as the 90% confidence interval of the group mean difference (*C* and *D*). Adjoining lines indicate data points from paired experiments. * $P < 0.05$, ** $P < 0.01$.

Study IV: The role of sphingomyelinase in disease-related loss of muscle function and mass

In study IV, the effects of sphingomyelinase on the force production, Ca^{2+}-handling, and transcriptional regulation of genes related to muscle atrophy processes were studied in intact muscle fibres. The activity of sphingomyelinase was measured in skeletal muscle from human heart failure and healthy age-matched subjects, and related to circulatory markers of inflammation and factors of known prognostic value in heart failure, previously reported by our lab for the current patient cohort (Rullman *et al*., 2020).

The results show that sphingomyelinase activity impaired SR Ca^{2+} release and reduced myofibrillar Ca^{2+} sensitivity to depress force in intact human muscle fibres (**Figure 3.7**).

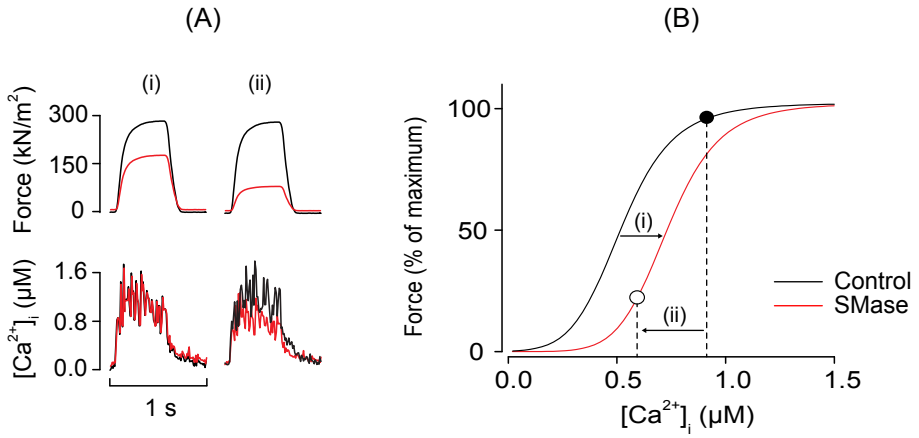

Figure 3.7. **Illustration of the effects of sphingomyelinase on muscle fibre force and Ca^{2+}-handling.** Sphingomyelinase-induced changes in tetanic force and $[Ca^{2+}]_i$ (*A*) and in the force-$[Ca^{2+}]_i$ relationship (*B*) in human intact muscle fibres. Sphingomyelinase activity reduced myofibrillar Ca^{2+} sensitivity (*i*) and decreased tetanic $[Ca^{2+}]_i$ (*ii*). SMase, sphingomyelinase.

In human muscle bundles, sphingomyelinase induced the transcription of genes of the ubiquitin-proteasome system, including essential subunits of the proteasome 20s core (PSMB5, 6, 7 and PSMA4), the 19s regulator (PSMC 1, 3, 4, 5 and PSMD1), polyubiquitin B and C (**Figure 3.8*A***). Meanwhile, transcripts of ribosomal proteins (**Figure 3.8*B***), factors implicated in muscle proliferation, and cellular locomotion were reduced by sphingomyelinase.

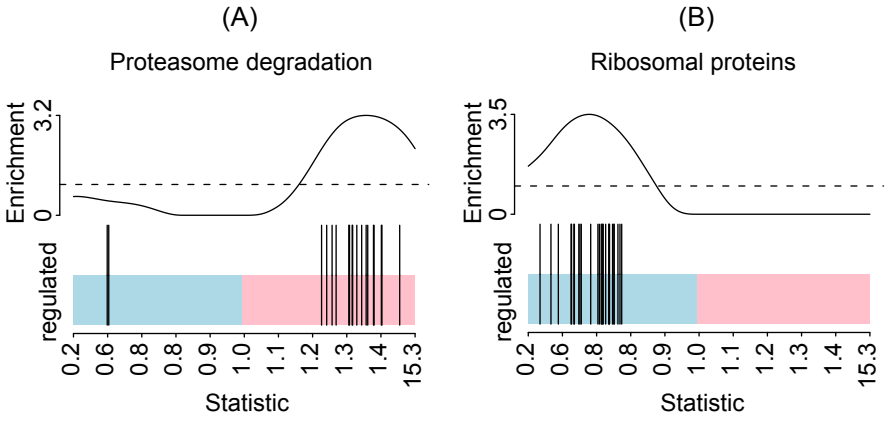

Figure 3.8. **Sphingomyelinase activity induced transcriptional changes promoting muscle atrophy in human intact muscle fibres.** Barcode-plots showing sphingomyelinase-induced changes in factors involved in proteasome degradation (*A*) and ribosomal proteins (*B*). In the lower panel each regulated gene of interest is depicted as a vertical line on the corresponding ratio (blue panel depicts down-regulated and red up-regulated genes) in treated *vs*. control-muscle. The upper panel depicts the enrichment of fold-changes between treated *vs*. control-muscle.

In human heart failure patients, the activity of intramuscular neutral sphingomyelinase (**Figure 3.9*A***), but not acid sphingomyelinase, was increased as compared to age-matched healthy controls. In the cohort of patients included in the longitudinal axis of the study, both neutral (**Figure 3.9*B***) and acid sphingomyelinase activity were found to increase with duration of disease. Intramuscular neutral sphingomyelinase activity positively correlated with circulatory markers of inflammatory activation and atrophy including TNF-RI and II, and markers previously demonstrated to carry prognostic value in this patient cohort (Rullman *et al*., 2020), including NT-proBNP, IGFBP7 and GDF15 (**Figure 3.9*C***).

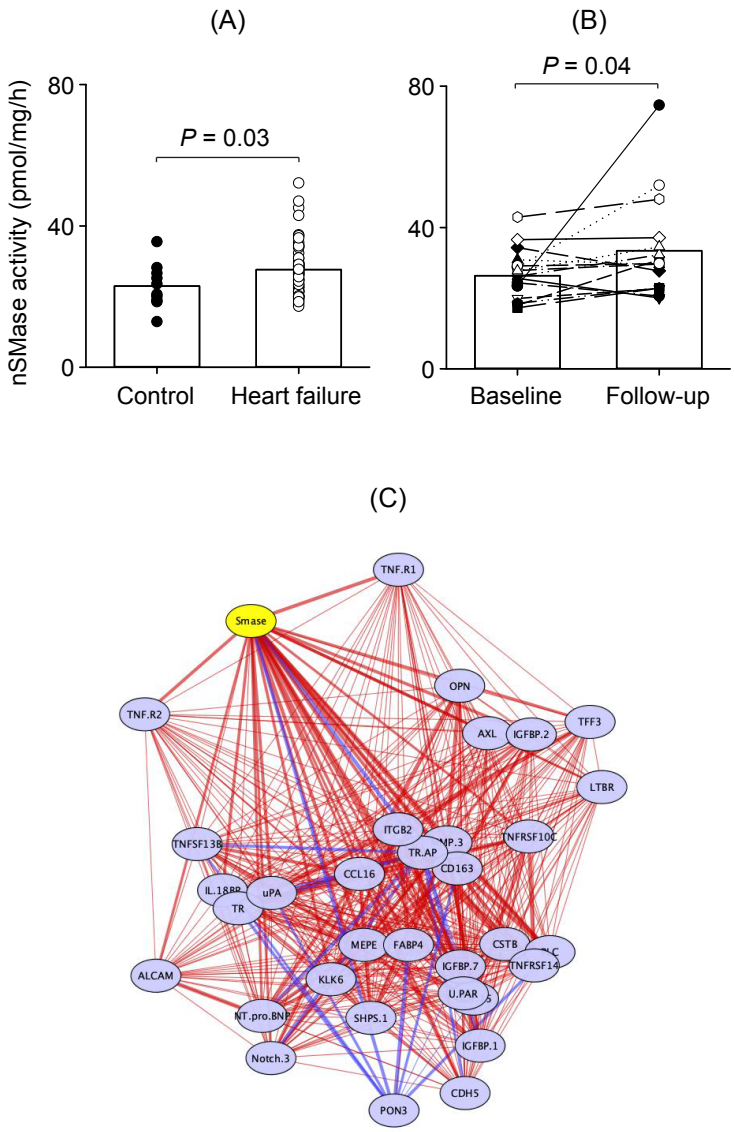

Figure 3.9. **Intramuscular neutral sphingomyelinase activity is elevated in human heart failure and correlates to circulatory markers of inflammation and muscle atrophy.** *A*, skeletal muscle nSMase activity in human heart failure patients and healthy age-matched controls. *C*, skeletal muscle nSMase activity in human heart failure patients at the time of the baseline biopsy (time of inclusion in the study) and at the follow-up biopsy. Mean follow-up time 2.5 (± 0.2) years. Bars indicate mean value and circles represent individual data points. *C*, correlation of skeletal muscle nSMase activity and serum protein quantities of factors analysed with targeted proteomics in human heart failure patients ($n = 61$) where edges denote correlation coefficients >0.3. nSMase, neutral sphingomyelinase.

8 Discussion and implications

This thesis investigated the cellular mechanisms underlying human muscle fatigue and disease-related muscle dysfunction, with a special focus on Ca^{2+}-regulated processes. Novel contributions include a mechanistic insight into the underlying causes of fatigue in human muscle fibres, the direct effects of vitamin D in muscle, and the role of sphingomyelinase in disease-induced muscle weakness and atrophy.

Ex vivo models of human skeletal muscle

At the time this thesis work commenced, manually dissected single fibres were arguable still the best model for mechanistic studies of muscle fibre functions. An obvious drawback of this model is the inferred problem of species differences, i.e., can we be sure that the findings in rodent muscles are representative of those in human muscles? A tentative approach to overcome this issue is the use of differentiated human myotubes. These can be obtained from minimally invasive muscle biopsies (Blau & Webster, 1981), and have been acclaimed traits that are similar to muscle fibres. In addition, they have been shown to retain donor-specific qualities (Berggren *et al*., 2007; Aas *et al*., 2013). The possibility to study disease-related changes in human muscle cells spurred our interest to investigate the utility of myotubes for studies of muscle fibre functions.

In study I, myotubes were found to develop some fibre-like qualities, but they did not develop functional excitation-contraction coupling. Hence, at least using the 2D culture protocol employed in study I, myotubes were found to be an inadequate model for studies of these muscle fibre functions. At the time of study I and in the years that followed, 3D cultures and continuous electrical stimulation were demonstrated to ameliorate the development of functional excitation-contraction coupling in myotubes (Madden *et al*., 2015; Rao *et al*., 2018; Khodabukus *et al*., 2019). Despite this, major differences in force production and Ca^{2+} transient kinetics were still apparent between these bioengineered myotubes and muscle fibres. Manually dissected single fibres from human intercostal muscles were therefore chosen in the following studies of adult muscle fibre functions in this thesis. However, the technical difficulties related to the dissection and the dependence of surgical procedures makes this a time-consuming and low-throughput model. Continuous efforts to develop *ex vivo* models of human myotubes amenable for studies of muscle fibre contraction and Ca^{2+}-handling are therefore anticipated and warranted.

Effects of vitamin D in human skeletal muscle

In study II, we found components of the vitamin D endocrine system were abundant in human muscle stem cells, while low to non-detectable in adult skeletal muscle. The apparent lack of the VDR in adult muscle fibres contrasts some previous reports in human and rodent skeletal muscle (Simpson *et al*., 1985; Costa *et al*., 1986; Bischoff *et al*., 2001; Pojednic *et al*., 2015). The reason for this disparity is likely due to differences in the antibody used to detect the VDR. Notably, when using the stringently validated VDR D-6 antibody, Wang and DeLuca (2011) reported that the VDR was un-detectable in skeletal muscle fibres, while confirming unspecific binding of other commercially available antibodies (Wang & DeLuca, 2011). While this does not exclude a direct role of vitamin D in muscle fibres, these findings suggest direct effects of vitamin D in human skeletal muscle to predominantly involve muscle stem cells.

Coherent with such a role, results in study II demonstrated a robust direct effect of vitamin D in human muscle stem cells: the biologically active $1\alpha,25(OH)_2D_3$, but not $25(OH)D_3$, inhibited human myoblast proliferation and differentiation. This marks a contribution to the literature, as previous studies have been limited to animal muscle stem cells (Garcia *et al*., 2011; Srikuea *et al*., 2012; Girgis *et al*., 2014a; Girgis *et al*., 2014b).

In order to maintain the skeletal muscle stem cell pool, part of the myoblast progeny must return to quiescence by events inhibiting myoblast proliferation and differentiation (Kuang *et al*., 2007). This process is controlled by the FOXO3 and Notch signalling pathways (Kuang *et al*., 2007; Bjornson *et al*., 2012; Gopinath *et al*., 2014), both of which we found to be modulated by vitamin D. Corroborating such an action, vitamin D deficiency has been suggested to contribute to the advent of sarcopenia in rodent muscles via diminished muscle stem cell proliferative and skeletal muscle regenerative potential by down-regulation of the Notch signalling pathway (Domingues-Faria *et al*., 2014). Notably, increased fibre size and strength has been reported in rat muscles electroporated with the vitamin D receptor (Bass *et al*., 2020). These effects were attributed partly to an increase in muscle stem cell activity.

Collectively, our results suggest that vitamin D plays a role in muscle stem cell self-renewal and quiescence, possibly by modulating the FOXO3 and Notch signalling pathways. This could be of importance for the overall functionality of the skeletal muscle tissue, especially in the ageing individual. The levels of the biologically active form of vitamin D is strictly regulated and well maintained despite very low levels of the prohormone 25(OH)D (Need *et al*., 2008). Impaired skeletal muscle function due to vitamin D deficiency is therefore likely uncommon. However, this will be of importance in conditions associated with a decline in the synthesis of the biologically active form of vitamin D, such as in chronic kidney disease, or in severe vitamin D deficiency.

Cellular mechanisms of fatigue in human muscle fibres

Study III highlighted that impaired SR Ca^{2+} release has a prominent role in the force loss during fatigue and in the early recovery period in human muscle fibres. This was in line with previous papers identifying reduced SR Ca^{2+} release as an important underlying cause of muscle fatigue in rodent fibres (Westerblad & Allen, 1991, 1993a). Impaired SR Ca^{2+} release is believed to stem from Ca^{2+}-P_i precipitation in the SR (Fryer *et al.*, 1995; Westerblad & Allen, 1996), in combination with an inhibition of the RyR1 channel caused by the depletion of cytosolic [ATP] and accumulation of free [Mg^{2+}] (Blazev & Lamb, 1999).

A distinct initial phase of increased tetanic [Ca^{2+}]$_i$ while tetanic force declines, previously described in rodent fibres, was observed in human fatigue-sensitive fibres. This early force reduction has been attributed primarily to accumulation of P_i that depresses maximal Ca^{2+}-activated force (Pate & Cooke, 1989; Millar & Homsher, 1990; Dahlstedt *et al.*, 2000). Considering the identical pattern seen in fatigue-sensitive human fibres, we suggest this to be the most likely cause also in human fibres.

Unlike in rodent fibres, a reduction in myofibrillar Ca^{2+} sensitivity did not contribute to fatigue in human muscle fibres. Intriguingly, an increase in myofibrillar Ca^{2+} sensitivity was seen in fatigue-resistant fibres. This implies that changes in myofibrillar Ca^{2+} sensitivity can contribute to the fatigue-resistance of slow-twitch human fibres. The underlying mechanisms of fatigue between human and rodent muscle fibres are illustrated in **Figure 8.1**.

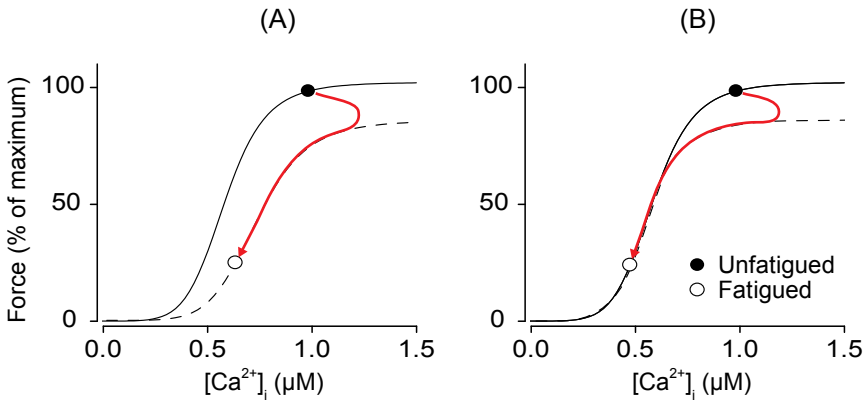

Figure 8.1. **Illustration of the cellular mechanisms of fatigue in rodent and human muscle fibres.** Contribution of changes in maximal Ca^{2+}-activated force, reduced SR Ca^{2+} release, and decreased myofibrillar Ca^{2+} sensitivity to fatigue in rodent (*A*) and human (*B*) fast-twitch fibres. Note that in rodent fibres (*A*) both a reduced SR Ca^{2+} release and decreased myofibrillar Ca^{2+} sensitivity contributes to the accelerated final decline in force, while in human fibres (*B*) this is caused exclusively by an impaired SR Ca^{2+} release. Force-[Ca^{2+}]$_i$ relation in unfatigued (solid line) and fatigued (dashed line) fibres.

Fatigue-induced changes in myofibrillar Ca^{2+} sensitivity have been investigated in human skinned fibres subjected to prior fatiguing stimulation *in vivo*. Results from these studies suggest that contractile-induced changes are multifaceted and can induce both an increased and decreased myofibrillar Ca^{2+} sensitivity of slow- and fast-twitch fibres (Hvid *et al*., 2013; Gejl *et al*., 2016; Lamboley *et al*., 2020). Contractile-induced RONS is one tentative factor involved in the modulation of myofibrillar Ca^{2+} sensitivity. Notably, RONS-mediated modifications of the fast troponin I isoform have been linked to both and increase and a decrease in myofibrillar Ca^{2+} sensitivity (Murphy *et al*., 2008; Mollica *et al*., 2012; Dutka *et al*., 2017). This was explained by a competitive action on the Cys134 residue of troponin I by different RONS-species, which could explain the reported diversity of changes in myofibrillar Ca^{2+} sensitivity. Additionally, phosphorylation of the regulatory myosin light chain of fast-twitch fibres has been acknowledged to increase myofibrillar Ca^{2+} sensitivity (Sweeney *et al*., 1993). An alternative hypothesis is that ADP and AMP accumulates in human fatigue-resistant fibres, due to the lower AMP deaminase and adenylate kinase activity in slow-twitch than in fast-twitch fibres (Borges & Essén-Gustavsson, 1989; Norman *et al*., 1994). Notably, ADP has been shown to increase the myofibrillar Ca^{2+} sensitivity of slow-twitch fibres (Macdonald & Stephenson, 2006).

Acidosis and human muscle fatigue

Coherent with findings in rodents, intracellular acidosis caused a decrease in myofibrillar Ca^{2+} sensitivity while tetanic $[Ca^{2+}]_i$ increased in unfatigued human fibres. These changes inferred under acidic conditions in unfatigued fibres are strikingly different to the fatigue-induced changes under control conditions. This indicates that intracellular acidosis does not occur during fatigue under the present conditions or, alternatively, that other contractile-induced changes completely offset the effects of acidosis. Owing to a lack of tissue, fatigue-induced changes in pH_i were not measured in any of the human fibres. Therefore, we cannot exclude the possibility that fatigue under the current experimental conditions results in a less marked drop in pH_i than what is seen *in vivo*. Notably, in rodent fibres fatigued by similar experimental protocols as in the present study, development of intracellular acidosis of a magnitude similar to that in human muscles fatigued *in vivo* has been reported (Sahlin *et al*., 1975; Sahlin *et al*., 1976; Bruton *et al*., 1998). Major differences in fatigue-induced changes in pH_i between the current experimental setting and the *in vivo* situation thus appear unlikely. To fully address these questions, future studies should include measurements of fatigue-induced changes in pH_i and $[Ca^{2+}]_i$ under control and acidic conditions, respectively.

In order to maintain tetanic force following a decrease in myofibrillar Ca^{2+} sensitivity, tetanic $[Ca^{2+}]_i$ must be adjusted accordingly. This will infer an increased energetic cost of force production due to the demand for energy-dependent removal of Ca^{2+} during relaxation. In analogy, fatigue is mitigated when myofibrillar Ca^{2+} sensitivity is increased (Cheng et al., 2019). It thus appears likely that acidosis would exacerbate fatigue by increasing the metabolic cost of force production. This may become evident when combined with other mechanisms affecting force production, as previously demonstrated under circumstances of severe acidosis combined with markedly elevated P_i concentrations (Cooke *et al*., 1988; Nelson *et al*., 2014; Nelson & Fitts, 2014; Sundberg *et al*., 2018). Repeated sprints may be such an example where severe acidosis is combined with profound changes in metabolites to further aggravate fatigue (**Figure 8.2**).

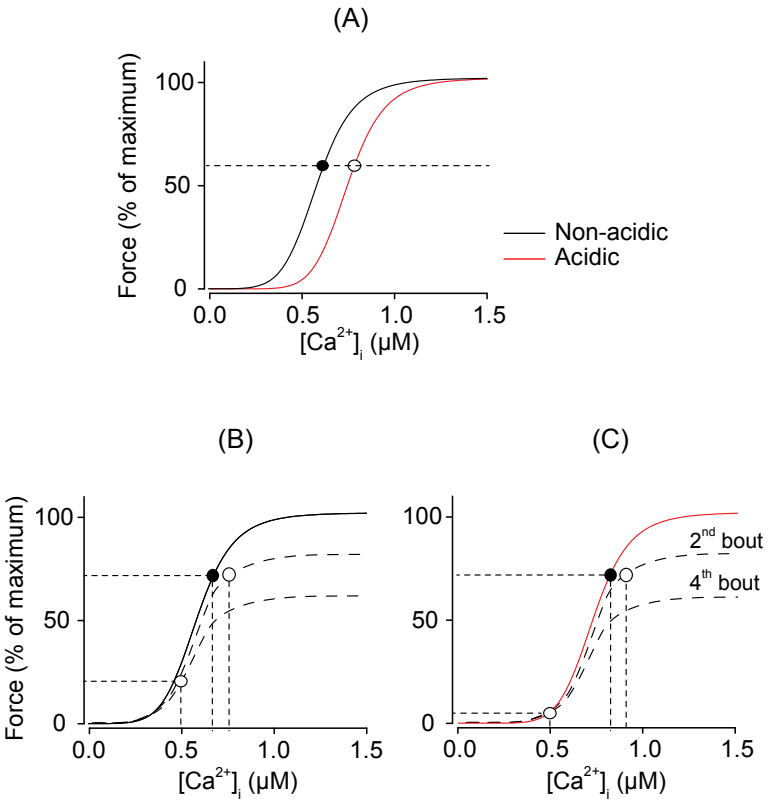

***Figure 8.2*. Tentative effects of acidosis on human muscle fatigue tolerance.** *A*, the myofibrillar Ca^{2+} sensitivity is reduced under acidic conditions, but this effect is offset by an increase in tetanic $[Ca^{2+}]_i$ in unfatigued human muscle fibres. *B* and *C*, illustration of the hypothetic response to repeated fatigue bouts in human muscle fibres under non-acidic (*B*) and acidic conditions (*C*).

The role of sphingomyelinase in disease-related loss of muscle function and mass

Study IV implicate elevated sphingomyelinase activity as an underlying cause of disease-related loss of muscle function and mass. The following findings support this notion: (*i*) sphingomyelinase decreased myofibrillar Ca^{2+} sensitivity and impaired SR Ca^{2+} release to depress force in intact muscle fibres, (*ii*) transcriptional changes promoting muscle atrophy processes were induced upon sphingomyelinase exposure in human fibres, (*iii*) the activity of intramuscular sphingomyelinase was elevated in heart failure compared to control individuals and (*iv*) positively correlated with circulating markers of inflammation, atrophy and factors carrying prognostic value in heart failure.

The effects of sphingomyelinase on force generation and myofibrillar Ca^{2+} sensitivity are in line with previous reports in rodent fibres (Ferreira *et al*., 2012; Empinado *et al*., 2014; Loehr *et al*., 2014; Bost *et al*., 2015). We report the first study of the effects of sphingomyelinase on SR Ca^{2+} release. Previous studies have been limited to chemically skinned fibres and muscle bundles, which do not allow such investigations (Place *et al*., 2010; Cheng & Westerblad, 2017). By demonstrating a depressive effect of sphingomyelinase on SR Ca^{2+} release, our data thus add novel insight into the force depression caused by sphingomyelinase.

In daily life, the ability to endure a physical activity at a set intensity or load over time (i.e. fatigue resistance) is dictated by the muscle strength and endurance. With all other variables set, if muscle strength declines this will demand that each contraction requires a higher α-motoneuron firing frequency in order to elevate $[Ca^{2+}]_i$ and to produce the required force. This will increase the energetic cost of each contraction and inevitably shorten the time to task failure, i.e. exhaustion. The muscle weakness caused by sphingomyelinase will thus be experienced as a decreased fatigue resistance in afflicted patients (**Figure 8.3**). In severe cases, the demands of daily life activities (e.g. rising up from a chair, walking up stairs) will demand near maximal Ca^{2+}-activated contractions and will be perceived as profound and debilitating muscle weakness and fatigue intolerance.

In human muscle fibres, transcriptional changes induced by sphingomyelinase included an up-regulation of genes involved in proteasome degradation and a down-regulation of genes encoding ribosomal proteins. The ubiquitin-proteasome is one of the two major proteolytic systems reported to be involved in muscle atrophy (Bonaldo & Sandri, 2013). Gene expression analyses of diverse clinical conditions associated with muscle atrophy have revealed common transcriptional changes of genes regulating the ubiquitin-proteasome system (Bodine *et al*., 2001; Gomes *et al*., 2001). These include an up-regulation of transcripts encoding ubiquitin, ubiquitin-conjugating enzymes, ubiquitin-protein ligases and several proteasome subunits (Lecker *et al*., 2004). Notably, several of these common

transcripts, including essential subunits of the 20s core and 19s regulatory proteasome components, as well as polyubiquitin C, were induced in human muscle fibres exposed to sphingomyelinase. Among the ribosomal proteins transcriptionally repressed following sphingomyelinase exposure were RPL6, a component of the 60s ribosomal subunit also known to be an intracellular partner of FGF2 (Shen *et al*., 1998). RPL6-FGF2 interaction is thought to promote cell survival (Sluzalska *et al*., 2020) and thus supressed RPL6 transcription may promote atrophy via cellular apoptosis. In light of the reported association of sphingomyelinase activity and muscle atrophy, e.g., in heart failure patients (Doehner *et al*., 2007), and our finding of elevated sphingomyelinase activity in skeletal muscle of heart failure patients, a decisive role of sphingomyelinase in disease-related muscle atrophy is suggested.

In skeletal muscle, TNFα acts through TNF-RI to activate neutral sphingomyelinase-3 (Moylan *et al*., 2014). Neutral sphingomyelinase-3 does not have a well-defined role in other organs and does not appear to be critical for general sphingolipid metabolism. Hence, it provides a tentative target for selective inhibition that could mitigate disease-related muscle dysfunction and muscle wasting. Given the emerging role of sphingomyelinase activation in various clinical conditions, and the recent introduction of inhibitors of other sphingomyelinase isoforms (Sala *et al*., 2020), this will be an interesting area of research to follow in the future.

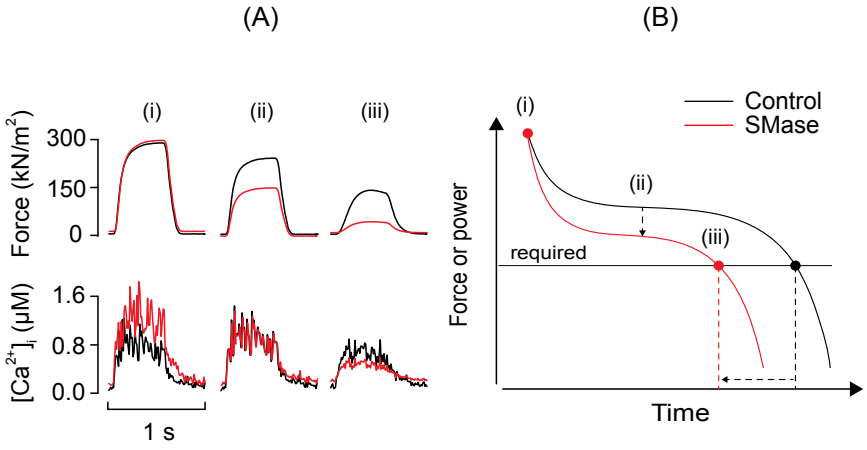

Figure 8.3. **Illustration of the effects of sphingomyelinase on human muscle force production, tetanic $[Ca^{2+}]_i$, and plausible effects on fatigue tolerance.** *A*, sphingomyelinase depresses force by reducing tetanic $[Ca^{2+}]_i$ and decreasing myofibrillar Ca^{2+} sensitivity. *B*, plausible effects of intracellular sphingomyelinase on the fatigue tolerance of human muscle fibres. *A* and *B*, sphingomyelinase reduces myofibrillar Ca^{2+} sensitivity so that larger increases in $[Ca^{2+}]_i$ are needed to attain maximal Ca^{2+}-activated force (*i*), while equimolar $[Ca^{2+}]_i$ produces less force than in controls (*ii*). The combined effects of reduced myofibrillar Ca^{2+} sensitivity and impaired SR Ca^{2+} release severely depresses force in fibres exposed to sphingomyelinase (*iii*).

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